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(54) Title: POTENTIATION OF COMPLEMENT AND COAGULATION INHIBITORY PROPERTIES OF C1-INHIBITOR.

(57) Abstract

Dextran sulphate is used to potentiate C1-esterase inhibitor selectively with respect to inhibition of complement and coagulation, but not with respect to inhibition of the contact and fibrinolytic systems. The C1-esterase inhibitor to be potentiated by the dextran sulphate may be endogenous C1-esterase inhibitor, or exogenous C1-esterase inhibitor which is to be administered together with or separate from the dextran sulphate. Use of the dextran sulphate, alone or together with C1-esterase inhibitor, in prophylactic or therapeutic treatment of inflammatory conditions, such as sepsis and myocardial infarction.

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Title: Potentiation of complement and coagulation inhibitory properties of C1-inhibitor

Field of the Invention

This invention is in the fields of immunology and biochemistry and describes a method to modify the inhibitory spectrum of C1-inhibitor, a major plasma inhibitor of multiple proteases of the complement, contact, fibrinolytic and coagulation plasma cascade systems. More specifically, it is demonstrated that inhibition of complement and clotting proteases by C1-inhibitor can be potentiated up to over 100-fold, without affecting its inhibitory properties towards fibrinolytic or contact system proteases. This potentiation is achieved by incubating C1-inhibitor with the synthetic sulfated polysaccharide dextran sulphate. Pharmaceutical compositions containing potentiated C1-inhibitor have considerable applications, for example as anti-inflammatory agent for the prophylactic or therapeutic treatment of sepsis or myocardial infarction.

Background of the Invention

Inflammatory reactions occur in the course of numerous human and animal diseases and are mediated by an array of so-called inflammatory mediators. Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press Ltd, 1992. Inflammatory mediators include activated monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, platelets and endothelial cells; cytokines; prostaglandins; leukotrienes; platelet activating factor; histamin and serotonin; neuropeptides; reactive oxygen species; and nitric oxide and related compounds.

Also the major plasma cascade systems, which include the coagulation, fibrinolytic, contact and complement

systems, contribute to inflammatory reactions since during activation of these systems fragments are generated, which have potent biological effects and are therefore considered to be inflammatory mediators. The plasma cascade systems each consist of a series of plasma proteins, most of which are synthesized by the liver and circulate in blood as inactive precursors, also called factors. Activation of the first factor of a system comprises conversion by limited proteolysis of the inactive, often single-chain precursor into a cleaved often two-chain active protein. This activated first factor subsequently activates, again by limited proteolysis, a number of inactive second factors, which in turn each activate a number of third factors and so on. This reaction pattern resembles a cascade. Excessive activation of the plasma cascade systems is regulated by the presence of a series of inhibitors including the multi-specific inhibitor α2-macroglobulin and the serine proteinase inhibitors (serpins) antithrombin III, α1-antitrypsin, α1-antichymotrypsin, α2-antiplasmin, C1-inhibitor, and others.

The complement system

The complement system constitutes one of the plasma cascade systems. Its physiological role is to defend the body against invading micro-organisms and to remove necrotic tissue and cellular debris.

The complement system can be activated via two pathways, a classical and an alternative pathway, which both can trigger activation of a common terminal pathway. Cooper N.R., 1985, Adv Immunol 37: 151; Muller-Eberhard H.J. et al., 1980, Adv Immunol 29: 1; Muller-Eberhard H.J., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press Ltd, p.33.

Activation of complement results in the generation of biologically active peptides, also known as the anaphylatoxins. These anaphylatoxins, in particular C3a and C5a, are chemotactic for neutrophils and able to aggregate, activate

and degranulate these cells. Vogt W., 1986, Complement 3: 177; Goldstein IM, 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press Ltd, p.63; Hugli TE, 1984, Springer Semin Immunopathol 7: 193. Furthermore, they may enhance vasopermeability, stimulate adhesion of neutrophils to endothelium, activate platelets and endothelial cells, and induce degranulation of mast cells and the production of vasoactive eicosanoids, thromboxane A2 and peptidoleukotrienes such as LTC4, LTD4 and LTE4 by mononuclear cells. Also the so-called terminal complement complexes (TCC), formed upon activation of the common pathway, have important biological effects including the capability to lyse target cells and, at sublytic concentrations, to induce cells to release mediators, such as cytokines, proteinases and eicosanoids. Muller-Eberhard H.J., 1986, Ann Rev Immunol 4: 503; Hansch GM, 1992, Immunopharmacol 24: 107. Finally, complement activation products may induce the expression of tissue factor by cells and thereby initiate and enhance coagulation. Osterud B et al., 1984, Haemostasis 14: 386; Hamilton KK et al., 1990, J Biol Chem 265: 3809. Thus, complement activation products have a number of biological effects, which may induce or enhance inflammatory reactions.

Activation of complement is considered to play an important role in the pathogenesis of a number of inflammatory disorders, including sepsis and septic shock; toxicity induced by the in vivo administration of cytokines or monoclonal antibodies (mAbs); immune complex diseases such as rheumatoid arthritis, systemic lupus erythematosus and vasculitis; multiple trauma; ischaemia-reperfusion injuries; myocardial infarction; and so on. The pathogenetic role of complement activation in these conditions is likely related in some way or another to the aforementioned biological effects of its activation products. Inhibition of complement activation may, therefore, add to the treatment of these conditions.

As just mentioned, complement can be activated via two different pathways, the classical and the alternative pathway. The latter will not be discussed here since C1-inhibitor is not known to have an effect on this pathway. Classical pathway activation starts with activation of the first component, which consists of a macromolecular complex of 5 proteins, one C1q, two C1r and two C1s proteins. The C1q protein of the C1 complex binds to an activator, for example immune complexes, which leads to activation of both C1r and both C1s subcomponents. Schumaker VN et al., 1987, Rev Immunol 5: 21; Cooper N.R., 1985, Adv Immunol 37: 151. During activation C1r and C1s are converted from single peptide-chain inactive proteins into two-chain active serine proteinases. The activated C1 complex then activates the complement factors C4 and C2, which together form the bi-molecular C4b,2a complex. Polley MJ et al., 1968, J Exp Med 128: 533; Kerr MA, 1980, Biochem J 189: 173. This complex then activates C3, the third component of complement, by cleaving it into the smaller fragment C3a and the larger C3b. The C4b,2a complex is hence called a C3-convertase.

Cleavage of C5 by a C5-convertase, which is generated by fixation of an additional C3b molecule to a C3-convertase, yields the anaphylatoxin C5a and nascent C5b, which latter together with C6 forms the bimolecular C5b,C6 complex, which in turn binds C7. The C5b,C6,C7 complex either inserts into a membrane or interacts with S protein. Interaction with S protein finally yields soluble membrane attack complexes (MAC). C5b,C6,C7 inserted into a membrane forms a receptor for C8. Subsequently, the tetramolecular C5b-8 complex will bind and polymerize C9, yielding fully assembled membrane-inserted MAC complexes, each consisting of the C5b-8 complex and one or more C9 molecules. Muller-Eberhard H.J., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press, p.33; Muller-Eberhard HJ, 1986, Annu Rev Immunol 4: 503.

Several plasma proteins can inhibit activation of the classical pathway of complement, notably, C1-inhibitor, C4-

binding protein and the serine-proteinase factor I. Muller-Eberhard H.J., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press, p.33; Schumaker VN et al., 1987, Ann Rev Immunol 5: 21; Cooper NR, 1985, Adv Immunol 37: 151. Of these, C1-inhibitor will be described in more detail below.

The contact system

The contact system consists of a set of proteins, which circulate in blood as inactive precursor proteins. The system is also known as the contact system of coagulation or the kallikrein-kinin system. Colman R.W., 1984, J Clin Invest 73: 1249; Kaplan A.P. et al., 1987, Blood 70: 1; Kozin F. et al., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press, p.103. The contact system constitutes one of the major plasma cascade systems, and is often regarded as one of the two pathways of clotting, the so-called extrinsic pathway of coagulation being the other.

Activation of the contact system starts with the binding of factor XII, also known as Hageman factor, to an activator. Subsequently, bound factor XII may become activated, during which process it is converted from a single-chain inactive into a two-chain active serine proteinase. Tans G. et al., 1987, Sem Thromb Hemost 13: 1. Activated factor XII then activates prekallikrein, that via its cofactor high molecular weight kininogen is bound to the activator, into the active serine proteinase kallikrein. Kallikrein in turn may activate bound but not yet activated factor XII (reciprocal activation). Factor XIIa may activate factor XI, which in turn can activate factor IX to start activation of coagulation. Cochrane C.G. et al., 1982, Adv Immunol 33: 290; Colman R.W., 1984, J Clin Invest 73: 1249; Kaplan A.P. et al., 1987, Blood 70: 1; Kozin F. et al., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New

York, Raven Press, p.103. Activation of the contact system is controlled by the same protein that also inhibits the classical complement pathway, C1-inhibitor, and which will be discussed below. During activation of the contact system several biologically active fragments are formed such as bradykinin, kallikrein and activated factor XII. These fragments may enhance activation and degranulation of neutrophils, increase vasopermeability and decrease vascular tonus. Colman R.W., 1984, J Clin Invest 73: 1249; Kozin F. et al., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press, p.103.

It is generally accepted that the contact system becomes activated in inflammatory conditions. Colman R.W., 1984, J Clin Invest 73: 1249; Kaplan A.P. et al., 1987, Blood 70: 1; Kozin F. et al., 1992, In: Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates, New York, Raven Press, p.103. However, its precise role in inflammation as well as that under physiological conditions is not well understood. Persons with a genetic deficiency of factor XII may have an increased risk for thromboembolic disease. This, together with its tissue type-plasminogen-like structure (Tans G. et al., 1987, Sem Thromb Hemost 13: 1), suggests that factor XII participates in fibrinolysis. In vivo observations on the contribution of factor XII to plasminogen activation in homo- and heterozygous factor XII deficient individuals are in agreement herewith. Levi M. et al., 1991, J Clin Invest 88: 1155.

Factor XI is often considered as a member of the contact system since in vitro it can be activated by factor XII. Kurachi K. et al., 1977, Biochemistry 16: 5831. It is a dimeric glycoprotein consisting of two identical polypeptide chains held together by a disulfide bond. Upon activation, each polypeptide chain can be cleaved at an internal peptide bond giving rise to disulfide linked heavy and light chains, the latter each containing one active site. Bouma B.N. et al., 1977, J Biol Chem 252: 6432; Van der Graaf F. et al.,

1983, J Biol Chem 258: 9669; Fujikawa K. et al., 1986, Biochemistry 25: 2417. The activity of each active site of factor XIa is regulated by plasma protease inhibitors including α1-antitrypsin, antithrombin III, C1-inhibitor, and α2-antiplasmin, each a member of the superfamily of serine protease inhibitors (serpins). Soons H. et al., 1987, Biochemistry 26: 4624-4629. Heck L.W. et al., 1974, J Exp Med 140: 1615; Damus P.S. et al., 1973, Nature 246: 355; Forbes C.D. et al., 1970, J Lab Clin Med 76: 809; Saito H. et al., 1979, Proc Natl Acad Sci USA 76: 2013. Initial studies suggested α1-antitrypsin to be the main inhibitor of factor XIa in plasma. Scott C.F. et al., 1982, J Clin Invest 69: 844. However, studies with enzyme-linked immunosorbent assays to quantitate complexes between factor XIa and its inhibitors in plasma demonstrated C1-inhibitor to be a major inhibitor of factor XIa. Wuillemin W.A. et al., 1995, Blood 85: 1517.

The in vivo role of factor XI may be unrelated to contact activation: recent studies have suggested that activation of factor XI may occur independently from factor XII via thrombin and contribute to activation of factor IX. Naito K. et al., 1991, J Biol Chem 266: 7353; Gailani D. et al., 1991, Science 253: 909. In this view factor XI acts to enhance thrombin generation, initially induced by the extrinsic pathway. Davie E.W. et al., 1991, Biochemistry 30: 10363; Broze Jr. G.J., 1992, Seminars Hematol 29: 159. This supposed role of factor XI in the coagulation system is consistent with clinical data that the only deficiency of a contact system protein, which results in a (mild) bleeding disorder, is that of factor XI. This, together with the lack of evidence that in vivo the contact system participates in the process of coagulation, raises serious doubts on whether factor XI should be considered as a contact system protein. Anyway, regardless the precise role of factor XI in the clotting mechanism, inhibition of factor XIa will attenuate coagulation, without the risk of a severe bleeding tendency as for example is induced by heparin-mediated potentiation of antithrombin III.

C1-inhibitor

C1-inhibitor, also known as C1-esterase inhibitor, refers to a protein that is present in blood and is the main inhibitor of the classical pathway of complement and of the contact system. C1-inhibitor can inhibit the activated form of the first component of complement and activated factor XII, and it is also a major inhibitor of kallikrein. Schapira M. et al., 1985, Complement 2: 111; Davis A.E., 1988, Ann Rev Immunol 6: 595; Sim R.B. et al., 1979, FEBS Lett 97: 111; De Agostini A. et al., 1984, J Clin Invest 73: 1542; Pixley R.A. et al., 1985, J Biol Chem 260: 1723; Schapira M. et al., 1982, J Clin Invest 69: 462; Van der Graaf F. et al., 1983, J Clin Invest 71: 149; Harpel P.C. et al., 1975, J Clin Invest 55: 593. Thus, C1-inhibitor regulates the activity of two plasma cascade systems, i.e. the complement and contact systems, that during activation generate biologically active peptides. C1-inhibitor is, therefore, an important regulator of inflammatory reactions. In addition, C1-inhibitor is a major inhibitor of activated factor XI. Meijers J.C.M. et al., 1988, Biochemistry 27: 959; Wuillemin W.A. et al., 1995, Blood 85: 1517. Considering the possible function of factor XI as discussed above, C1-inhibitor should therefore also be considered as a coagulation inhibitor. Also tissue-type plasminogen activator and plasmin are inhibited to some extent by C1-inhibitor, although this inhibitor is not the major inhibitor of these proteinases. Harpel P.C. et al., 1975, J Clin Invest 55: 149; Booth N.A. et al., 1987, Blood 69: 1600. C1-inhibitor should therefore also be considered as a (weak) fibrinolytic inhibitor.

C1-inhibitor has been purified from plasma at large scale and used for clinical application, particularly in the treatment of heriditary angioedema, a disease caused by a genetic deficiency of C1-inhibitor. Furthermore, administration of C1-inhibitor has been claimed to have beneficial effects in other diseases as well, such as systemic inflammatory responses in mammals [Fong S., 1992, WO 92/22320 (Genentech Inc)], and of complications of severe burns,

pancreatitis, bone marrow transplantation, cytokine therapy and the use of extracorporeal circuits [Eisele B. et al., 1994, DE-A-4227762 (Behringwerke AG)]. The present invention relates to these therapeutical applications of C1-inhibitor in that it provides a novel method to enhance the inhibitory activity of C1-inhibitor, and hence reduces the amount of C1-inhibitor needed for these therapies.

Full-length genomic and cDNA coding for C1-inhibitor has been cloned. Bock S.C. et al., 1986, Biochemistry 25: 4292; Carter P.E. et al., 1988, Eur J Biochem 173: 163. Functional recombinant C1-inhibitor protein has been expressed in COS cells and found to be similar to the plasma protein. Eldering E. et al., 1988, J Biol Chem 263: 11776. Several variants of recombinant C1-inhibitor with amino acid mutations at the P1 and the P3 and/or P5 position of the reactive centre as well as variants isolated from patients with hereditary angioedema have been expressed in the same system. Eldering E. et al., 1988, J Biol Chem 263: 11776; Eldering E. et al., 1993, J Biol Chem 267: 7013; Eldering E. et al., 1993, J Clin Invest 91: 1035; Patent Cetus Corp, US617920; Davis A.E. et al., 1992, Nature Genetics 1: 354; Eldering E. et al., 1995, J Biol Chem 270: 2579; Verpy et al., 1995, J Clin Invest 95: 350.

C1-inhibitor belongs to a superfamily of homologous proteins known as the serine-proteinase inhibitors, also called serpins. Travis J. et al., 1983, Ann Rev Biochem 52: 655; Carrel R.W. et al., 1985, Trends Bioch Sci 10: 20. On sodium dodecylsulphate polyacrylamide gels C1-inhibitor has an apparent molecular weight of approximately 105 kD. Its plasma concentration is about 270 mg/l. Schapira M et al., 1985, Complement 2: 111; Nuijens JH et al., 1989, J Clin Invest 84: 443. C1-inhibitor is an acute phase protein whose levels may increase up to 2-fold during uncomplicated infections and other inflammatory conditions. Kalter ES et al., 1985, J Infect Dis 151: 1019. The increased synthesis of C1-inhibitor in inflammatory conditions is most probably meant to protect the organism against the deleterious effects of (intravascular) activation of the complement and

contact systems during acute phase reactions. In patients with rheumatoid arthritis the synthetic rate of C1-inhibitor may increase up to 2.5 times the normal rate. Woo et al., 1985, Clin Exp Immunol 61: 1. Metabolic studies with radiolabeled C1-inhibitor in normal volunteers have yielded a fractional catabolic rate (FCR) of 2.5% of the plasma pool per hour and an apparent plasma half-life time of clearance of about 20 hours. Woo et al., 1985, Clin Exp Immunol 61: 1; Quastel M. et al., 1983, J Clin Invest 71: 1041.

The serpins share a similar mechanism of inhibition, which is characterized by forming stable bi-molecular complexes with the proteinase to be inhibited. In these complexes the active site of the proteinase is bound to the so-called reactive centre of the serpin and hence rendered inactive. Travis J. et al., 1983, Ann Rev Biochem 52: 655. Like other serpins C1-inhibitor inhibits proteinases by forming stable complexes with these proteinases, which are rapidly cleared from the circulation. De Smet B.J.G.L. et al., 1993, Blood 81: 56. Serpins have specificity for certain proteinases and this specificity is in part determined by the amino acid sequence of the reactive centre.

The activity of serpins may be influenced by glycosaminoglycans, a heterogeneous group of macromolecular sulphated glycoconjugates linked to a protein core. Kjellen L. et al., 1991, Annu Rev Biochem 60: 443; Poole A.R., 1986, J Biochem 236: 1; Bourin M.-C. et al., 1993, Biochemical J 289: 313. This group includes the physiological compounds heparin, heparan sulfate and dermatan sulfate. Poole A.R., 1986, J Biochem 236: 1. For example, heparan sulfate and heparin-like molecules are endothelial cell-associated glycosaminoglycan in the vascular bed. Ausprunk D.H. et al., 1981, Am J Pathol 103: 353; Marcum J.A. et al., 1985, Biochem Biophys Res Comm 126: 365; Ihrcke N.S. et al., 1993, Immunology Today 14: 500. Glycosaminoglycans have been claimed to have anti-metastatic and/or anti-inflammatory activities based on their properties to inhibit endoglycosidases, particularly heparinase. Parish C.R. et al., 1988,

WO 88/05301 (Australia University). This effect of glycosaminoglycans is unrelated to the present invention.

Its enhancing effects on the function of antithrombin underlie the therapeutical use of heparin. Furthermore, sulphated polysaccharides may exert additional anticoagulant activities in the presence of lipoprotein-associated coagulation inhibitor (LACI), which effect has been patented for therapeutic application. Tze-Chein Wun, 1992, EP-A-0473564 (Monsanto Company). The semisynthetic sulphated polysaccharide dextran sulphate has less enhancing effects on antithrombin III then heparin, although it may potentiate other inhibitors of coagulation such as protease nexin-1 (PN-1). Scott R.W., 1991, WO 91/05566 (Invitron Corp.). These effects of sulphated polysaccharides on clotting inhibitors are unrelated to the present invention, which is dealing with the interaction of dextran sulphate and C1-inhibitor. The heparin-antithrombin III interaction is probably the best studied example of glycosaminoglycan-enhanced function of a serpin. However, a number of studies have also shown that glycosaminoglycans, in particular heparin, may also potentiate the function of other serpins including C1-inhibitor: In kinetic assays with purified proteins heparin has been shown to potentiate the inhibition of C1s by C1-inhibitor 15- to 35-fold, whereas the inhibition of activated C1 or C1r is less enhanced. Rent R. et al., 1976, Clin Exp Immunol 23: 264; Sim R.B. et al., 1980, Biochim Biophys Acta 612: 433; Caughman G.B. et al., 1982, Mol Immunol 19: 287; Nilsson T. et al., 1983, Eur J Biochem 129: 663; Lennick M. et al., 1986, Biochemistry 25: 3890; Hortin G.L. et al., 1991, Immunol Invest 20: 75. This enhanced interaction of C1s occurs at the expense of an increased proteolytic inactivation of C1-inhibitor. Weiss V. et al., 1983, Hoppe-Seyler's Z Physiol Chem 364: 295. In addition to these effects on C1-inhibitor heparin has multiple other effects on the complement system such as inhibiting effects on the binding of C1q to an activator, on the activity of C1-esterase and on the formation of the classical C3-convertase. Raepple E. et al., 1976, Immunochemistry 13:

251; Loos M. et al., 1976, Immunochemistry 13: 257; Strunk R. et al., 1976, Clin Immunol Immunopathol 6: 248. Heparin might, therefore, be considered as a therapeutic complement inhibitor. However, the complement-inhibiting effects of heparin are observed at concentrations at least one order higher than those required for anticoagulant effects, and using such doses in vivo carries the unacceptable risk of bleeding. To reduce its anticoagulant properties a N-desulfated, N-acetylated form of heparin has been developed, which preparation has been shown to possess significant complement inhibitory properties. Weiler J.M. et al., 1992, J Immunol 148: 3210; Friedrichs G.S. et al., 1994, Circ Res 75: 701. However, this does not obviate another disadvantage of the use of heparin (or any other glycosaminoglycan), i.e., that it has to be purified from animal sources.

The mechanism by which glycosaminoglycans potentiate C1-inhibitor towards inhibition of its target proteases C1s and factor XIa is not known. However, in analogy to what is known for heparin-accelerated inhibition of thrombin by antithrombin III, several mechanisms are postulated: (I) Glycosaminoglycans may induce a conformational change in the inhibitor, rendering it more active; (II) Glycosaminoglycans may work as a template on which inhibitor and target protease may assemble; (III) Glycosaminoglycans may neutralize positive charges either on the inhibitor or on the protease or both, thereby allowing a more easy interaction. Evans D.L. et al., 1992, Biochemistry 31: 12629; Bode W. et al., 1994, Fibrinolysis 8: 161; Potempa J. et al., 1994, J Biol Chem 269: 15957. Which one of these mechanism(s) applies to the observed glycosaminoglycan-induced enhancement of C1-inhibitor function remains to be shown in further studies.

In the present invention the synthetic sulphated polysaccharide dextran sulphate is used to enhance the inhibitory activity of C1-inhibitor. Dextran sulphate and related compounds may be effective inhibitors of human immune deficiency virus type 1. De Clercq E.D.A. et al., 1988, EP-A-0293826 (Stichting Rega V.Z.W.). In addition,

dextran sulphate may be useful for the treatment of arteriosclerosis. Herr D., 1988, EP-A-0276370 (Knoll AG). These effects are unrelated to the present invention. Furthermore, high molecular weight species of dextran sulphate, but not low molecular weight species, are able to enhance auto-activation of factor XII of the contact system. Samuel M. et al., 1992, J Biol Chem 267: 19691.

Summary of the Invention

It has now been found that inhibitory properties of C1-inhibitor, a major inhibitor of various complement, clotting, contact system and fibrinolytic proteases, can be modified by incubation with a semisynthetic polyanionic compound, the sulphated polysaccharide dextran sulphate, yielding a C1-inhibitor selectively potentiated up to over 100-fold regarding its complement and clotting inhibitory properties. Therefore, the present invention contemplates a pharmaceutical composition containing C1-inhibitor with selectively enhanced function, that can be used prophylactically or therapeutically to inhibit activation of complement and/or coagulation in vivo. The pharmaceutical composition comprises C1-inhibitor and dextran sulphate species. Exemplary compositions may contain C1-inhibitor derived from human plasma or any other biological source, or recombinant C1-esterase inhibitor, or mutants derived therefrom. Exemplary compositions may also contain dextran sulphate of varying molecular weight, or any other synthetic polyanionic compound with comparable effects.

The invention will be more fully understood after a consideration of the following description of the invention.

Brief Description of the Drawings

Figure 1. Influence of glycosaminoglycans or DXS on the amidolytic activity of factor XIa. The amidolytic activity of factor XIa was determined as the initial change in absorbance at 405 nm at 37°C using the chromogenic substrate S-2366 at a final concentration of 0.4 mmol/l in a buffer containing 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, and

0.1 % (wt/vol) Tw. The effect of different amounts of DXS MW 500,000 (solid circles), DXS MW 5,000 (triangles), heparin (open circles), heparan sulfate (solid squares), or dermatan sulfate (open squares) was tested. Results are expressed as the percentage of the activity of 1 nmol/l factor XIa, in the absence of any glycosaminoglycans, remaining after addition of varying amounts of different glycosaminoglycans (μg/ml, final concentrations).

Figure 2. Kinetics of the inactivation of factor XIa by C1-inhibitor in the absence of glycosaminoglycans or DXS. Factor XIa (final concentration 6 nmol/l) was incubated at 37°C with different concentrations of C1-inhibitor in 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, 0.1 % Tw. At various times, aliquots were removed and assayed for residual amidolytic activity of factor XIa. (Panel A) Inactivation of factor XIa was assessed in the presence of C1-inhibitor at 0 (solid circles), 0.32 (open circles), 0.64 (solid squares), 0.96 (open squares) or 1.28 (plus signs) μmol/l. The natural logarithm of residual factor XIa amidolytic activity was plotted against time. (Panel B) The pseudo first-order rate constants (k, min^{-1}) were calculated from the slopes of the plots shown in panel A and plotted as a function of the C1-inhibitor concentration. The slope of the line represents the second order rate constant (k_2, min^{-1}, M^{-1}).

Figure 3. Kinetics of the inactivation of factor XIa by C1-inhibitor in the presence of glycosaminoglycans or DXS. Factor XIa (final concentrations 3 to 8 nmol/l) was incubated at 37°C with C1-inhibitor (final concentration 0.32 μmol/l) in 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, 0.1 % Tw, and the pseudo first-order rate constants were determined as described in legend to Figure 2. Results are expressed as the potentiation factor of the inhibition of factor XIa by C1-inhibitor in the presence of varying amounts of DXS MW 500,000, DXS MW 5,000, heparin, heparan sulfate or dermatan sulfate, compared with the inhibition rate in the absence of glycosaminoglycans or DXS.

Figure 4. Pseudo first-order rate constants of factor XIa inhibition by C1-inhibitor in the presence of glycosaminoglycans or DXS. The pseudo first-order rate constants were determined as described in legend to Figure 2, in the presence of varying concentrations of C1-inhibitor and in the presence of DXS MW 500,000, DXS MW 5,000, heparin, heparan sulfate or dermatan sulfate, or in the absence of glycosaminoglycans or DXS. The slope of the lines represents the second order rate constants (k_2, min^{-1}, M^{-1}).

Figure 5. Inhibition of C1s by C1-inhibitor in the presence of various glycosaminoglycans or DXS. C1s at a final concentration of 3 nmol/l was incubated with C1-inhibitor (final concentration 15 nmol/l) and various glycosaminoglycans (each tested at 10 µg/ml) in phosphate buffered saline (PBS)-0.05 % Tw, containing the chromogenic substrate S2314 at a final concentration of 0.8 mmol/l at 37°C. The change in absorbance at 405 nm in time is shown.

Figure 6. Dose-response of the enhancing effect of DXS MW 500,000 on the inhibition of C1s by C1-inhibitor. Conditions used are the same as described in Figure 5.

Figure 7. Dose-response of the enhancing effect of DXS MW 5,000 on the inhibition of C1s by C1-inhibitor. Conditions used are the same as described in Figure 5.

Figure 8. Inhibition by DXS MW 500,000 of complement activation in recalcified plasma by aggregated human IgG. Citrated (10 mmol/l, final concentration) blood was recalcified by adding 10 mM $CaCl_2$ (final concentration). After 15 min at 37°C a clot had formed, which was removed by centrifugation for 10 min at 2,000 x g at 4°C. One vol of recalcified plasma was then incubated with one vol veronal buffered saline containing aggregated human IgG at a concentration of 5 mg/ml for 20 min at 37°C. Complement activation during this incubation was then measured by assessing the generation of C1s-C1-inhibitor complexes, C4 and C3 activation products (C4b/C4bi/C4c and C3b/C3bi/C3c, respectively). Aggregated IgG was prepared as described. Hack C.E. et al., 1981, J Immunol 127: 1450. C1s-C1-inhibitor complexes and C3 and C4 activation products

were measured as previously described. Nuijens J.H. et al., 1989, J Clin Invest 84: 443; Wolbink G.J. et al., 1993, J Immunol Meth 163: 67. Results (mean and standard deviation of 3 experiments) are shown as % inhibition, 0% being the generation of activation products in the absence of DXS, 100% being the generation of complement activation products in the absence of aggregated IgG and DXS.

Figure 9. Inhibition by DXS MW 5,000 of complement activation in recalcified plasma by aggregated human IgG. The experiment was performed similarly as the one described in Figure 8, except that DXS MW 5,000 was used.

Figure 10. Inhibition by heparin of complement activation in recalcified plasma by aggregated human IgG. The experiment was performed similarly as that described in Figure 8, except that heparin was used.

Figure 11. Inhibition by N-acetyl-heparin of complement activation in recalcified plasma by aggregated human IgG. The experiment was performed similarly as the one described in Figure 8, except that N-acetyl-heparin was used.

Detailed Description of the Invention

Several patents/patents applications and scientific articles are referred to below that discuss various aspects of the materials and methods used to realize the invention. It is intended that all of the references be entirely incorporated by reference.

The kernel of the present invention is the realization that C1-inhibitor, a major inhibitor of complement, clotting, contact system and fibrinolytic proteases in plasma can be modified regarding its inhibitory spectrum by the semisynthetic compound dextran sulphate (DXS): the inhibitory properties of C1-inhibitor towards complement and coagulation systems are potentiated up to over 100-fold, whereas those towards contact and fibrinolytic systems are not affected. Virtually every method to modify the inhibitory function of C1-inhibitor by DXS is intended to come into the scope of this invention. Potentiating effects of glycosaminoglycans on the inhibition of C1s have been des-

cribed previously (see section "Background of invention"). However, these glycosaminoglycans are obtained from animal sources and to a varying extent also potentiate antithrombin III and heparin cofactor II. Low doses of these glycosaminoglycans are used in a clinical setting to treat thromboembolic diseases. To obtain inhibition of complement in patients, doses of heparin of at least one order higher are needed, which have the unacceptable risk of bleeding. The advantages of the present invention are: a) DXS has stronger enhancing effects on the inhibition of factor XIa and C1s than any glycosaminoglycan, as is illustrated below; b) only the larger forms of DXS may have some stimulating effects on antithrombin III and treatment with the low MW forms of this compound, therefore, does not have the risk of bleeding tendency; and c) DXS is a semisynthetic compound that can be produced in large quantities, whereas glycosaminoglycans such as heparin are purified from animals.

To more clearly define the present invention, it will be described in three sections. The first section describes the effects of DXS on the inhibition of target proteases factor XIa, factor XIIa, kallikrein and C1s by C1-inhibitor in purified systems. Results obtained with glycosaminoglycans are also given for comparison. The second section describes the effects of DXS on complement activation in plasma. The effects of heparin and N-acetyl-heparin, glycosaminoglycans sometimes used as complement inhibitors, are also given for comparison. The third section describes the application of DXS in therapeutical compositions containing C1-inhibitor.

<u>The effects of DXS on the inhibition of target proteases by C1-inhibitor in purified systems</u>

In this section the effects of DXS on the inhibition of target proteases factor XIa, factor XIIa, kallikrein and C1s by C1-inhibitor are presented. The type of experiments shown is the determination of pseudo-first order and second order rate constants, which constants describe the kinetics of the inhibition of target proteases by C1-inhibitor, and the

effects of DXS on these rate constants. The determination of rate constants for the inhibition of factor XIa by C1-inhibitor will be shown in detail, whereas that of the constants describing the inhibition of kallikrein, factor XIIa or C1s will be described more briefly. The effects of various glycosaminoglycans on the rate constants is also shown to illustrate that DXS is more potent in enhancing C1-inhibitor than any glycosaminoglycan. Finally, in case of factor XIa the effects of DXS or glycosaminoglycans on the inhibition by antithrombin III, α2-antiplasmin and α1-antitrypsin are also shown as these inhibitors significantly contribute to the inhibition of factor XIa in plasma.

Dextran sulfate (MW 500,000, sulfur content 17%) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; dextran sulfate (MW 5,000), heparan sulfate (from bovine intestinal mucosa) and soybean-trypsin inhibitor (SBTI, type I-S) from Sigma Chemical Co., St.Louis, MO; unfractionated heparin (1 U/ml corresponding to 7 μg/ml) from Kabi Vitrum, Stockholm, Sweden; dermatan sulfate (chondroitin sulfate B). Hexadimethrine bromide (Polybrene) was from Janssen Chimica, Beerse, Belgium; Tween-20 (Tw) from J.T. Baker Chemical, Phillipsburg, NJ. The chromogenic substrates Glu-Pro-Arg-p-nitroanilide (S-2366; factor XIa substrate) and H-D-Pro-Phe-Arg-p-nitroanilide (S-2302; factor XIIa and kallikrein substrate) were from Chromogenix, Mölndal, Sweden; H-D-Val-Ser-Arg-p-nitroanilide (S-2314; C1s substrate) from Kabi Diagnostica (Stockholm, Sweden).

Purified human factor XIa was obtained from Kordia Laboratory Supplies, Leiden, The Netherlands, and was stored at -70°C in 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, 0.1% (wt/vol) Tw. This preparation was made by incubating factor XI with factor XIIa, after which factor XIIa was removed by absorption onto a corn trypsin inhibitor column. Factor XIa preparation migrated as a single band at 160 kD on non-reducing, and as two bands at 50 and 30 kD, respectively, on reducing SDS/10-15% (wt/vol)-polyacrylamide gel electrophoresis. Monoclonal antibody (mAb) OT-2, which is directed against the light chain of activated factor XII

and blocks its catalytic activity (Dors D.M. et al., 1992, Thromb Haemost 67: 644) was added to the factor XIa preparation (80 µg/ml final concentration) to block traces of contaminating factor XIIa. Factor XIa concentrations were expressed as the molar concentration of the 80 kD subunits. Purified human α-factor XIIa was obtained from Kordia Laboratory Supplies, Leiden, The Netherlands. Kallikrein, β-factor XIIa and C1s were purified as described (Nuijens J.H. et al., 1987, Thromb Haemost 58: 778; Nuijens J.H. et al., 1987, Immunology 61: 387). Purified C1-inhibitor preparations were obtained from Behringwerke AG (Marburg, Germany) and from the department of Development of plasma products from our institute (CLB), α1-antitrypsin, α2-antiplasmin and antithrombin III were from Calbiochem (La Jolla, CA).

Amidolytic activity of factor XIa was determined in wells of microtiterplates (Greiner GmbH, Frickenhausen, Germany) by using the chromogenic substrate S-2366 at a final concentration of 0.4 mmol/l in a buffer containing 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, and 0.1 % (wt/vol) Tw (total volume of 200 µl). The initial change in absorbance at 405 nm (δA) was measured at 37°C using a Titertek twinreader (Flow Laboratories, Irvine, UK).

Glycosaminoglycans and DXS may affect directly the amidolytic activity of kallikrein. Tankersley D.L. et al., 1983, Blood 62: 448. Heparin, heparan sulfate or dermatan sulfate had no measurable effect on the amidolytic activity of factor XIa, whereas DXS MW 500,000, but not DXS MW 5,000 dose-dependently inhibited this activity up to 50% (Fig. 1). In further experiments, results obtained with DXS were corrected for this effect.

Factor XIa and inhibitors were incubated in the presence or absence of glycosaminoglycans or DXS in 0.5 ml polypropylene tubes at 37°C with 0.1 mol/l Tris-HCl, pH 7.4, 0.14 mol/l NaCl, 0.1 % (wt/vol) Tw as a buffer. Before incubation the various components of the mixtures were prewarmed at 37°C for 5 min. After addition of prewarmed factor XIa (final concentrations 3 to 8 nmol/l) to the

reaction mixtures, 10 µl aliquots were removed at various times and residual amidolytic activity of factor XIa was assessed by diluting in 190 µl buffer and substrate as described above. The observed δA/min, which was constant during the time of measurement, was converted to percentage of maximum activity by comparison with the δA/min of the sample containing factor XIa and glycosaminoglycan but no protease inhibitor. The kinetics of the inhibition were studied under pseudo first-order conditions with the inhibitors in 13 to 210-fold molar excess over factor XIa. Inactivation of factor XIa by C1-inhibitor indeed appeared to follow first-order kinetics under pseudo first-order conditions, as was concluded from the straight lines obtained when the natural logarithm of residual factor XIa amidolytic activity was plotted against time (Fig. 2A). Under these conditions, the equation $\ln(E/E_o)=-k \times t$, where E_o is the initial concentration of factor XIa, and E the concentration of remaining factor XIa at time t, describes the inhibitory kinetics (Soons H., 1987, Biochemistry 26: 4624). According to this equation, the values of the apparent first-order rate constants, k, were calculated from the slopes of these lines and were found to be directly proportional to the C1-inhibitor concentrations (Fig. 2B). Therefore, inhibition was found to be second-order, in agreement with previous studies. Soons H., 1987, Biochemistry 26: 4624. The rate constant describing the reaction was calculated by linear regression analysis and found to be 1.8×10^3 M^{-1} s^{-1}. The inhibition of factor XIa by C1-inhibitor in the presence of various amounts of dextran sulfate, heparin, heparan sulfate or dermatan sulfate also appeared to be first-order under pseudo first-order conditions. However, the rate constants increased with increasing amounts of the glycosaminoglycans (Fig. 3). DXS MW 500,000 and DXS MW 5,000 appeared to be more potent in enhancing the inhibition of factor XIa by C1-inhibitor than any of the physiological glycosaminoglycans tested (Fig. 4). Similar experiments were performed with the other inhibitors of factor XIa except that the equation

$k_1 = k_2 \times [C1\text{-}inhibitor]$ was used to calculate second order rate constants. Again, straight lines were obtained in a semilogarithmic plot of the residual factor XIa amidolytic activity against time demonstrating that the reaction was first-order. The values of the apparent second-order rate constants were calculated and are given in Table I. Each rate constant was determined at least twice, the variation between the different determinations was 9.6 ± 0.5 % (mean ± standard error of mean). Thus, though various glycosaminoglycans potentiated the inhibition of factor XIa by C1-inhibitor, the semisynthetic compound DXS was the compound that best potentiated C1-inhibitor regarding inhibition of factor XIa, i.e. up to over 100-fold. DXS also potentiated inhibition of factor XIa by antithrombin III (ATIII), but this effect was not greater than 5-fold, and it was also much weaker than that of heparin on AT III (Table I). The inhibition of factor XIa by α2-antiplasmin (a2AP) or α1-antitrypsin (a1AT) was hardly enhanced by DXS.

Table I. Second-order rate constants for the inactivation of factor XIa by C1-inhibitor (C1Inh), α1-antitrypsin (a1AT), α2-antiplasmin (a2AP), and antithrombin III (ATIII) in the presence of various glycosaminoglycans (GAG) or DXS

	($10^3 M^{-1}s^{-1}$)					
	no GAG	DXS[1]	DXS[2]	Hep*	HS*	DS*
C1Inh	1.8	210	160	85	42	6
α1AT	0.1	0.02	nd	0.06	0.08	0.06
α2AP	0.43	0.19	nd	0.51	0.57	0.65
ATIII	0.32	1.54	nd	4.4	1.27	1.24

[1] DXS MW 500,000 [10 μg/ml, final concentration]; [2] DXS MW 5,000 [10 μg/ml]; * Hep, heparin [50 U/ml]; HS, heparan sulfate [1 mg/ml]; DS, dermatan sulfate [1 mg/ml]; nd = not determined.

In an analogous way as described above, the effects of glycosaminoglycans and DXS on the inhibition of factor XIIa

or kallikrein by C1-inhibitor were investigated. No potentiation of C1-inhibitor was observed in these experiments (Table II).

Table II. Second-order rate constants for the inactivation of α-factor XIIa, β-factor XIIa and kallikrein by C1-inhibitor in the presence of various glycosaminoglycans (GAG) or DXS

	($10^3 M^{-1}s^{-1}$)				
	no GAG	DXS[1]	DXS[2]	HS*	DS*
α-FXIIa:	8.0	3.1	7.2	6.8	10.3
β-FXIIa:	9.8	5.4	1.7	8.2	11.9
kallikrein	25.5	22.1	19.4	24.5	26.0

[1] DXS MW 500,000 [125 μg/ml, final concentration]; [2] DXS MW 5,000 [125 μg/ml]; *HS, heparan sulfate [1 mg/ml]; DS, dermatan sulfate [1 mg/ml].

Thus, in spite of their enhancing effects on the inhibition of factor XIa by C1-inhibitor, DXS and glycosaminoglycans hardly had an effect, if any, on the inhibition of factor XIIa or kallikrein by C1-inhibitor.

The inhibition of C1s was analyzed using second order conditions. It appeared that the various glycosaminoglycans also potentiated the inhibition of C1s by C1-inhibitor. In Fig. 5 it is shown that DXS MW 500,000 best potentiates the inhibitory activity of C1-inhibitor (15 nM) on the amidolytic activity of C1s (3 nM) by C1-Inh (15 nM). Fig. 6 shows that this effect of DXS is optimal at DXS concentration of 10-20 μg/ml. Similar results were obtained with DXS MW 5,000 (see Fig. 3). Compiling these data yields second order rate constants of inhibition given in Table III.

Table III. Second-order rate constants for the inactivation of C1s by C1-inhibitor in the presence of various glycosaminoglycans (GAG)

GAG*	concentration#	($10^5 M^{-1}s^{-1}$)
blank	--	0.453
DXS 500,000	100 µg/ml	58.75
DXS 5,000	100 µg/ml	34.05
heparin	50 U/ml	26.24
N-ac-heparin	1 mg/ml	4.856
HS	1 mg/ml	8.755
DS	1 mg/ml	13.43
CSA	1 mg/ml	2.509
CSC	1 mg/ml	3.606

* DXS, dextran sulfate; HS, heparan sulfate; DS, dermatan sulfate; CSA/CSC, chondroitin sulphate A/C. # final concentration.

Thus, the experiments shown in this section indicate that the inhibition of C1s or factor XIa by C1-inhibitor can be potentiated by incubating C1-inhibitor with DXS, whereas inhibition of the contact system is not affected.

The effects of DXS on complement activation in plasma

Examples presented in this and the following section are meant to further illustrate the invention, and are not to be considered as limiting the scope of the invention. For example, variation in the source, type, or method of producing DXS species; different assays; different labels and/or signals; test supports of different materials and configurations may be employed without departing from the scope of the present invention.

The effects of DXS on the inhibition of complement by C1-inhibitor in serum may be tested by adding DXS to fresh human serum, followed by incubation at 37°C of the mixture with complement activators such as aggregated IgG, cobra venom factor, E.coli bacteria or zymosan. After this

incubation EDTA is added to prevent further activation and the mixture is tested for the presence of complement activation products such as C3a, C4a, C5a, C3b/bi/c, C4b/bi/c or C5b-C9. Assays for these complement activation products are well known in the art and can be obtained commercially. The preferred assays are those described by Hack C.E. et al., 1988, J Immunol Meth 108: 77; Hack C.E. et al., 1990, J Immunol 144: 4249; Nuijens J.H. et al., 1989, J Clin Invest 84: 443; and Wolbink G.J. et al., 1993, J Immunol Meth 163: 67.

As is shown in Fig. 8 and Fig. 9 DXS MW 500,000 as well as DXS MW 5,000 both significantly inhibited complement activation in serum by aggregated IgG: Both DXS species at a concentration of about 100-200 μg/ml nearly completely inhibited the generation of activated C4 and C3 in serum by the classical pathway activator aggregated IgG. In addition, DXS MW 500,000, but not DXS MW 5,000, also inhibited the generation of C1s-C1-inhibitor complexes, probably reflecting a direct effect of DXS MW 500,000 on the binding of C1q to aggregated IgG. The effects of heparin and N-acetyl-heparin were explored in similar experiments. As is shown in Fig. 10 heparin inhibited complement activation in serum by aggregated IgG similarly as DXS MW 5,000. In contrast, N-acetyl-heparin appeared to be a weaker complement inhibitor than heparin or DXS (Fig. 10). Effects of this heparin-species with reduced anticoagulant properties on the generation of activated C3 were hardly observed, whereas inhibition of C4 activation was not complete unless concentrations of 1 mg/ml were tested.

The effects of DXS on 1 U of purified C1-inhibitor were directly compared with the effects of increasing C1-inhibitor concentrations by assessing the effects of DXS-treated C1-inhibitor with those of a dose-response curve in a CH50 determination. To this, 1 U of C1-inhibitor preincubated with DXS, or various concentrations of C1-inhibitor without DXS, were added to recalcified plasma, and the CH50 titer of the mixtures were determined. The results, shown in Table IV, indicate that the decrease of CH50 titer upon addition

of high doses of C1-inhibitor, i.e., up to 135 U, was only moderate, i.e. from 44 to 27 U/ml. A similar effect was observed with 1 U of C1-inhibitor potentiated with DXS.

Table IV. Comparison of the effect of 1 U of C1-inhibitor potentiated with DXS with those of untreated C1-inhibitor on the hemolytic activity of recalcified plasma as determined by CH50 assay

plasma plus* (Units/ml)	CH50 titer
buffer	44
DXS 5,000 (100 μg/ml)	32
DXS 500,000 (100 μg/ml)	25
C1-Inh (1 U)/DXS 5,000 (100 μg/ml)	29
C1-Inh (1 U)/DXS 500,000 (100 μg/ml)	26
C1-Inh (1 U)/DXS 5,000 (10 μg/ml)	38
C1-Inh (1 U)/DXS 500,000 (10 μg/ml)	33
C1-Inh (5 U)	44
C1-Inh (15 U)	43
C1-Inh (45 U)	37
C1-Inh (135 U)	27

* DXS, dextran sulfate; C1-Inh, C1-inhibitor

Thus, the experiments described in this section indicate that DXS is able to potentiate C1-inhibitor in serum and to reduce the generation of complement activation products.

Application of DXS in therapeutical compositions containing C1-inhibitor

In the preferred embodiment of the invention, the therapeutic composition contains plasma-derived C1-inhibitor as the active ingredient, for example as prepared according to Voogelaar E.F. et al., 1974, Vox Sang. 26: 118. The virus safety of this preparation is guaranteed by the addition of hepatitis B-immunoglobulin and a heat treatment of the

freeze-dried preparation in the final container. Brummelhuis H.G.J. et al., 1983, Vox Sang. 45: 205, Tersmette et al., 1986, Vox Sang. 51: 239. C1-inhibitor is prepared from human plasma, depleted of vitamin K-dependent coagulation factors, according to a procedure which involves the following purification steps: 1) the starting plasma is 1 to 10 diluted with sterile destilled water; 2) the diluted plasma is incubated with DEAE-Sephadex A50 (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 2 g/kg, for 60 minutes at 8-10°C; 3) the DEAE-Sephadex is collected and washed with 150 mM sodium chloride, pH 7.0, and eluted with 10 mM trisodium citrate, 2 M sodium chloride, pH 7.0; 4) ammonium sulphate is added to the eluate to yield a final concentration of 50%, v/v; 5) after centrifugation at 13,000 rpm, ammonium sulphate is added to the supernatant to yield a final concentration of 65%, v/v; 6) the precipitate is collected by centrifugation and dissolved in 10 mM trisodium citrate, pH 7.0; 7) a diafiltration is performed to remove the ammonium sulphate and to concentrate the solution to a protein concentration of 40-50 mg/ml; 8) after the addition of Hepatitis B immunoglobulin (0.4 IU/ml), the solution is filtered through a 0.22 μm filter, dispensed in vials and freeze-dried; 9) the freeze-dried product is heat-treated for 72 hours at 60°C.

In the preferred embodiment of the invention, C1-inhibitor is mixed with DXS (for example, 100 μg per Unit of C1-inhibitor), incubated for one hour; and then administered by intravenous injection.

Claims

1. A pharmaceutical composition comprising a dextran sulphate species which selectively potentiates C1-esterase inhibitor with respect to inhibition of complement and coagulation but not with respect to inhibition of the contact and fibrinolytic systems, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein said dextran sulphate species is low molecular weight dextran sulphate.

3. The pharmaceutical composition of claim 2, wherein said dextran sulphate species is dextran sulphate having a molecular weight of about 5,000.

4. The pharmaceutical composition of claim 1, wherein said dextran sulphate species is dextran sulphate having a molecular weight of about 500,000.

5. The pharmaceutical composition of claim 1, further comprising C1-esterase inhibitor.

6. The pharmaceutical composition of claim 5, wherein said C1-esterase inhibitor is selected from the group consisting of C1-esterase inhibitor purified from plasma, C1-esterase inhibitor purified from biological material other than plasma, recombinant C1-esterase inhibitor, and a mutant of recombinant C1-esterase inhibitor.

7. The pharmaceutical composition of claim 5, wherein said C1-esterase inhibitor is selected from the group consisting of C1-esterase inhibitor purified from human plasma, C1-esterase inhibitor purified from biological human material other than plasma, human recombinant C1-esterase inhibitor, and a mutant of human recombinant C1-esterase inhibitor.

8. The pharmaceutical composition of claim 5, wherein said C1-esterase inhibitor and said dextran sulphate species are chemically linked to eachother.

9. The pharmaceutical composition of claim 1 or claim 5, comprising said dextran sulphate species in an amount which is effective to selectively potentiate C1-esterase inhibitor with respect to inhibition of complement and coagulation but not with respect to inhibition of the contact and fibrinolytic systems.

10. The pharmaceutical composition of claim 1 or claim 5, for use as an anti-inflammatory composition.

11. The pharmaceutical composition of claim 1 or claim 5, for the prophylactic or therapeutic treatment of sepsis or myocardial infarction.

12. A method of a prophylactic or therapeutic treatment of a mammal, which method comprises administration to said mammal of an effective amount of a dextran sulphate species which potentiates C1-esterase inhibitor selectively with respect to inhibition of complement and coagulation, but not with respect to inhibition of the contact and fibrinolytic systems.

13. The method of claim 12, further comprising administration to said mammal of a physiologically effective amount of C1-esterase inhibitor.

14. The method of claim 13, wherein said dextran sulphate species and said C1-esterase inhibitor are administered in the form of a physical mixture, or chemically linked to eachother, or in separate compositions.

15. A dextran sulphate species for use in a method of a prophylactic or therapeutic treatment of a mammal to potentiate C1-esterase inhibitor selectively with respect to inhibition of complement and coagulation, but not with respect to inhibition of the contact and fibrinolytic systems.

16. Use of a dextran sulphate species for preparing a pharmaceutical composition for specifically potentiating C1-esterase inhibitor with respect to inhibition of complement and coagulation, but not with respect to inhibition of the contact and fibrinolytic systems.

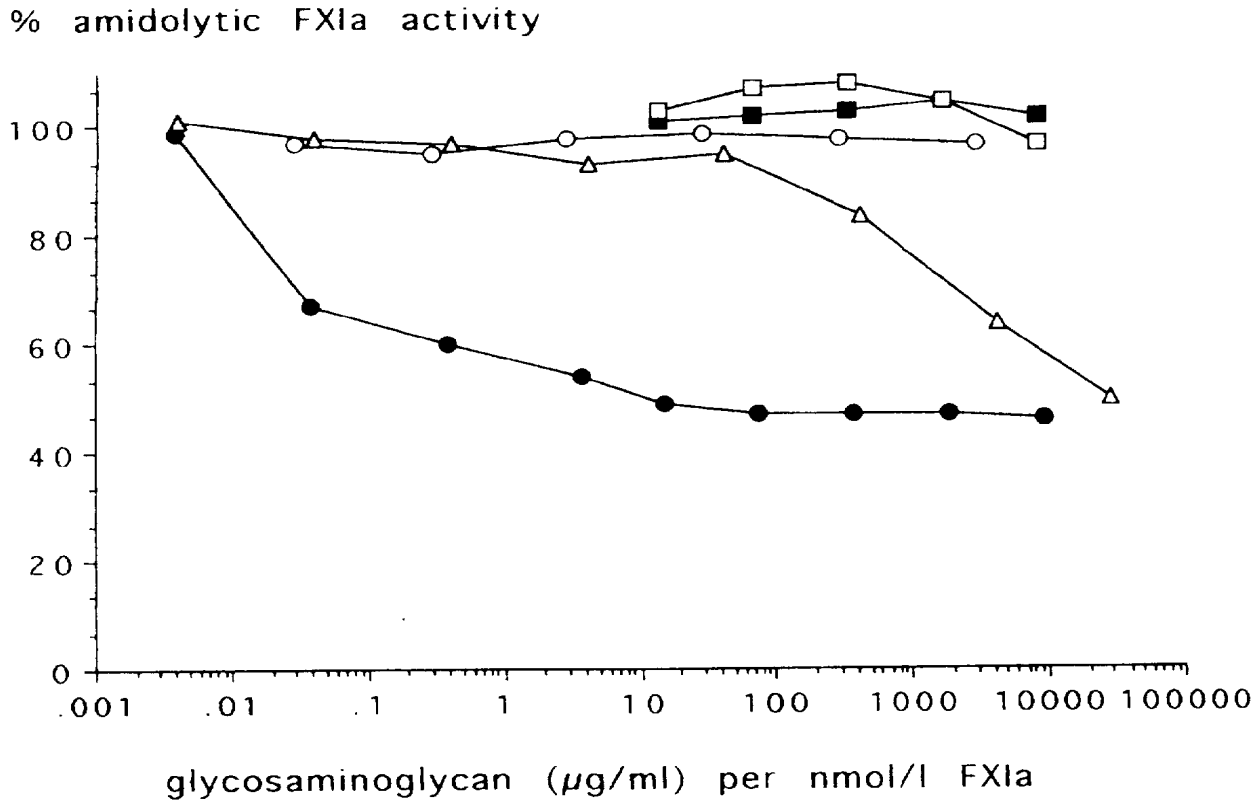
% amidolytic FXIa activity
100
80
60
40
20
0
.001
.01
.1
1
10
100
1000
10000
100000
glycosaminoglycan (μg/ml) per nmol/l FXIa

FIG. 1

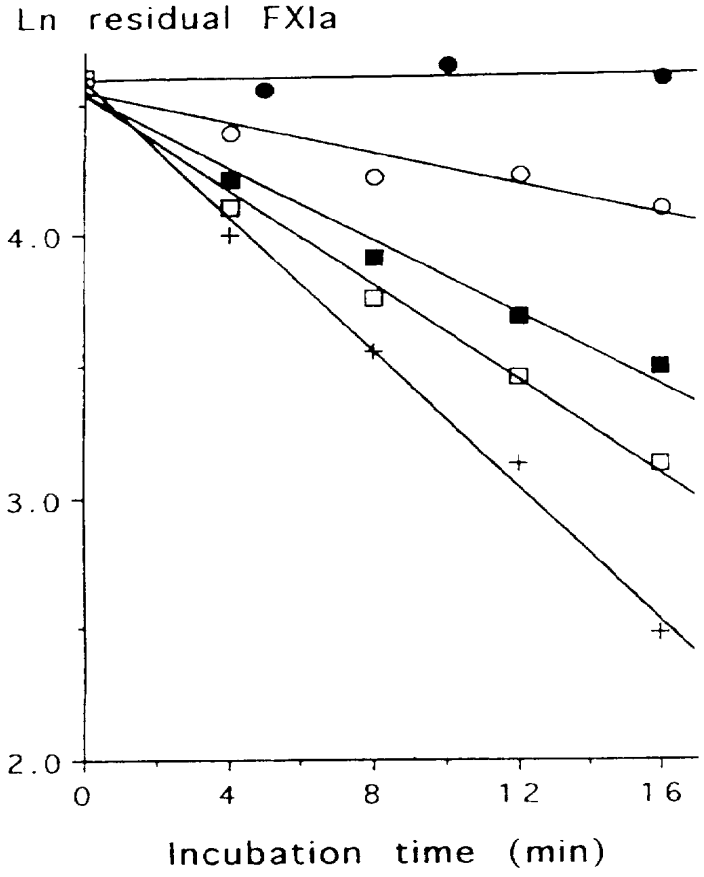

FIG. 2A

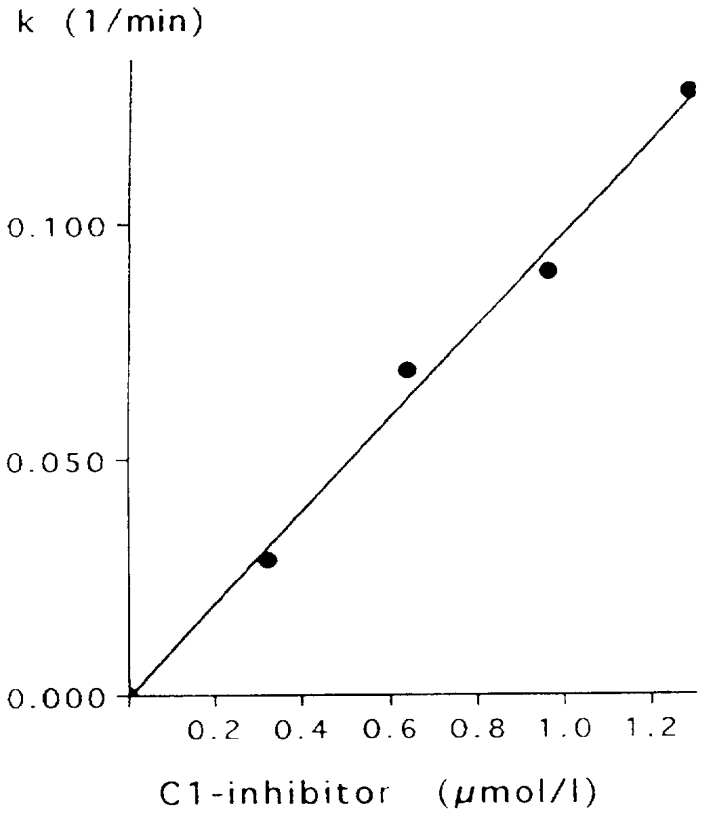

FIG. 2B

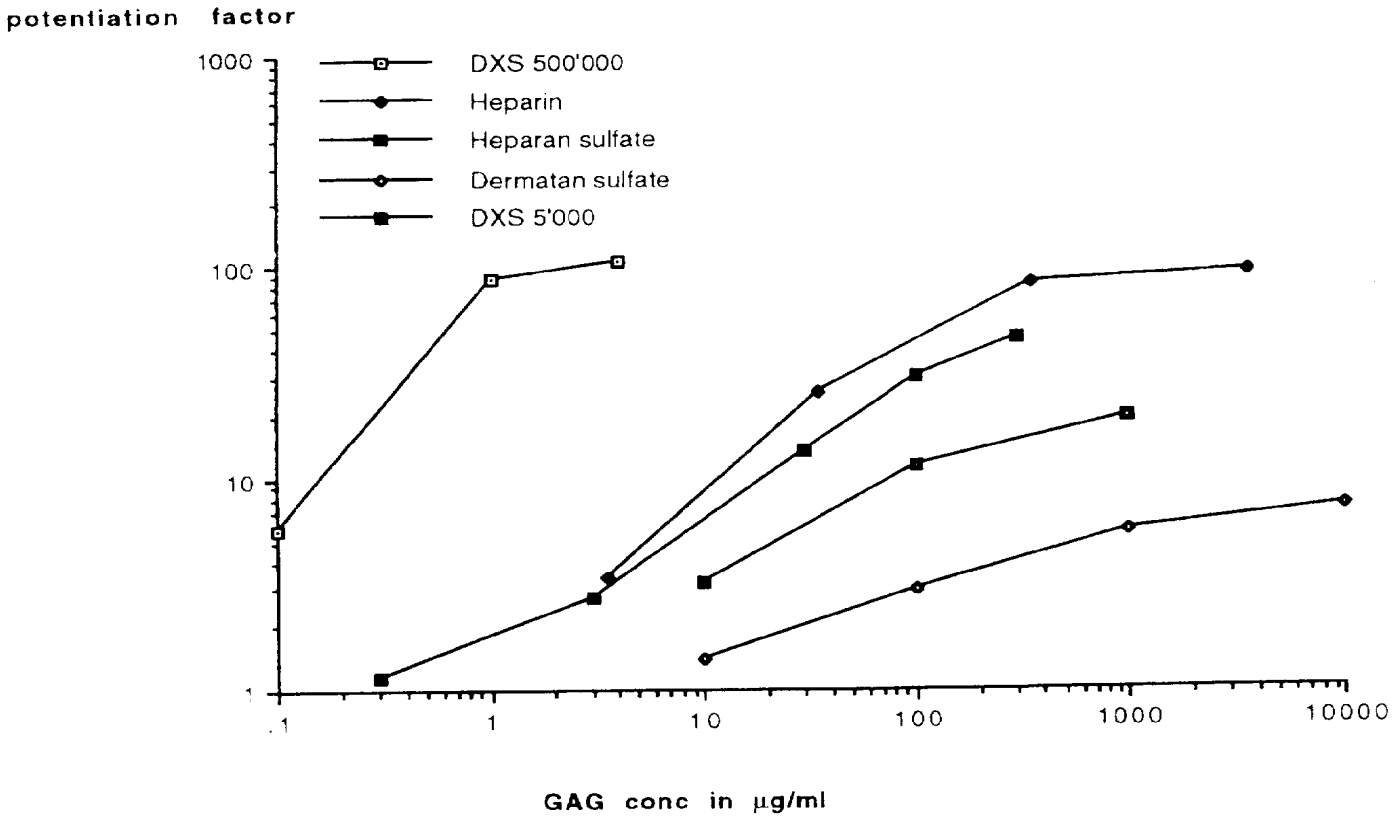
potentiation factor
1000
100
10
1
DXS 500'000
Heparin
Heparan sulfate
Dermatan sulfate
DXS 5'000
.1
1
10
100
1000
10000
GAG conc in µg/ml

FIG. 3

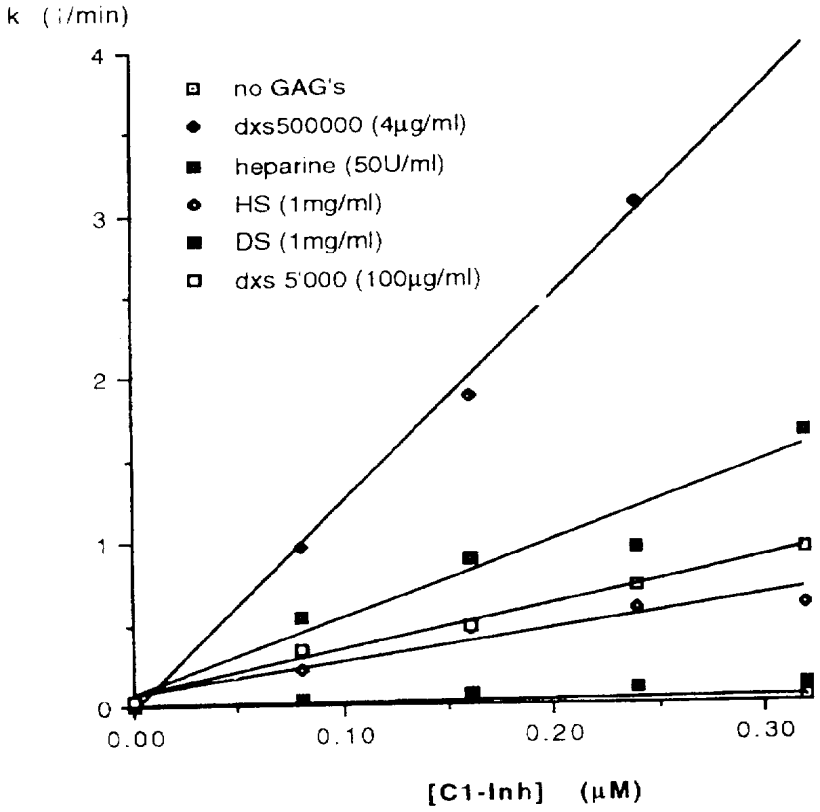
k (i/min)
4
3
2
1
0
no GAG's
dxs500000 (4µg/ml)
heparine (50U/ml)
HS (1mg/ml)
DS (1mg/ml)
dxs 5'000 (100µg/ml)
0.00
0.10
0.20
0.30
[C1-Inh] (µM)

FIG. 4

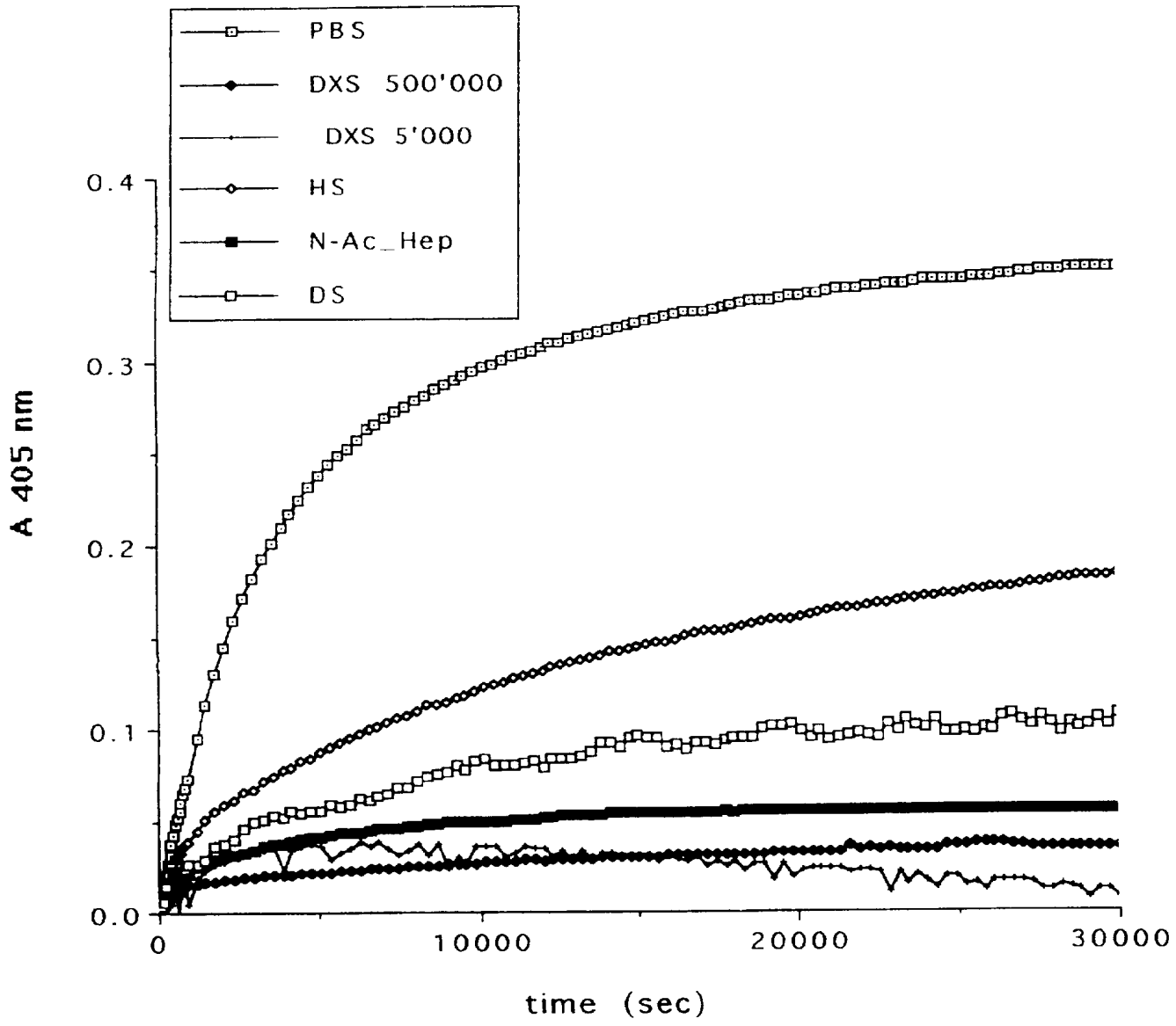
PBS
DXS 500'000
DXS 5'000
HS
N-Ac_Hep
DS
A 405 nm
0.0
0.1
0.2
0.3
0.4
0
10000
20000
30000
time (sec)

FIG. 5

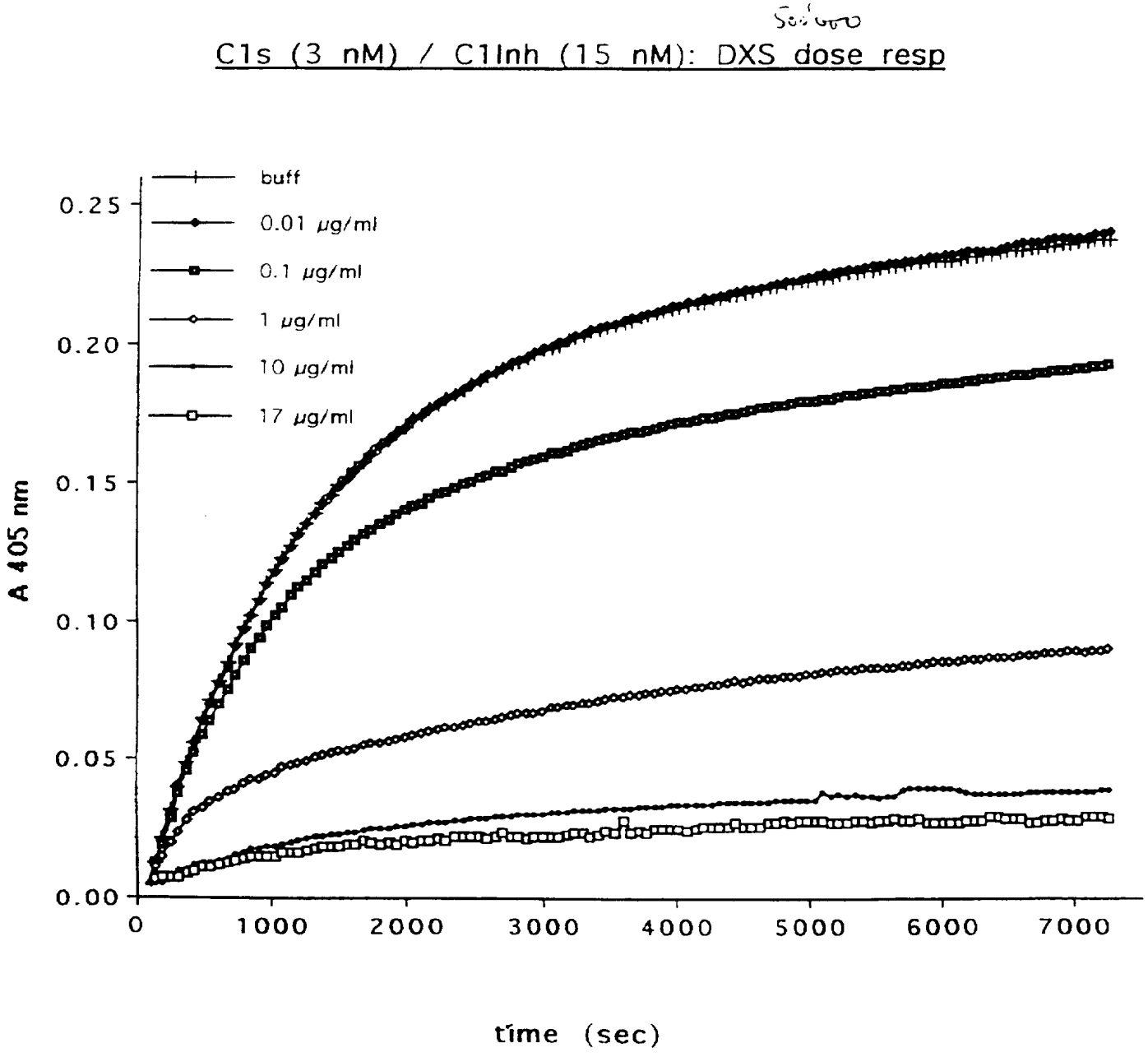
C1s (3 nM) / C1Inh (15 nM): DXS dose resp
buff
0.01 µg/ml
0.1 µg/ml
1 µg/ml
10 µg/ml
17 µg/ml
A 405 nm
0.25
0.20
0.15
0.10
0.05
0.00
0
1000
2000
3000
4000
5000
6000
7000
time (sec)

FIG. 6

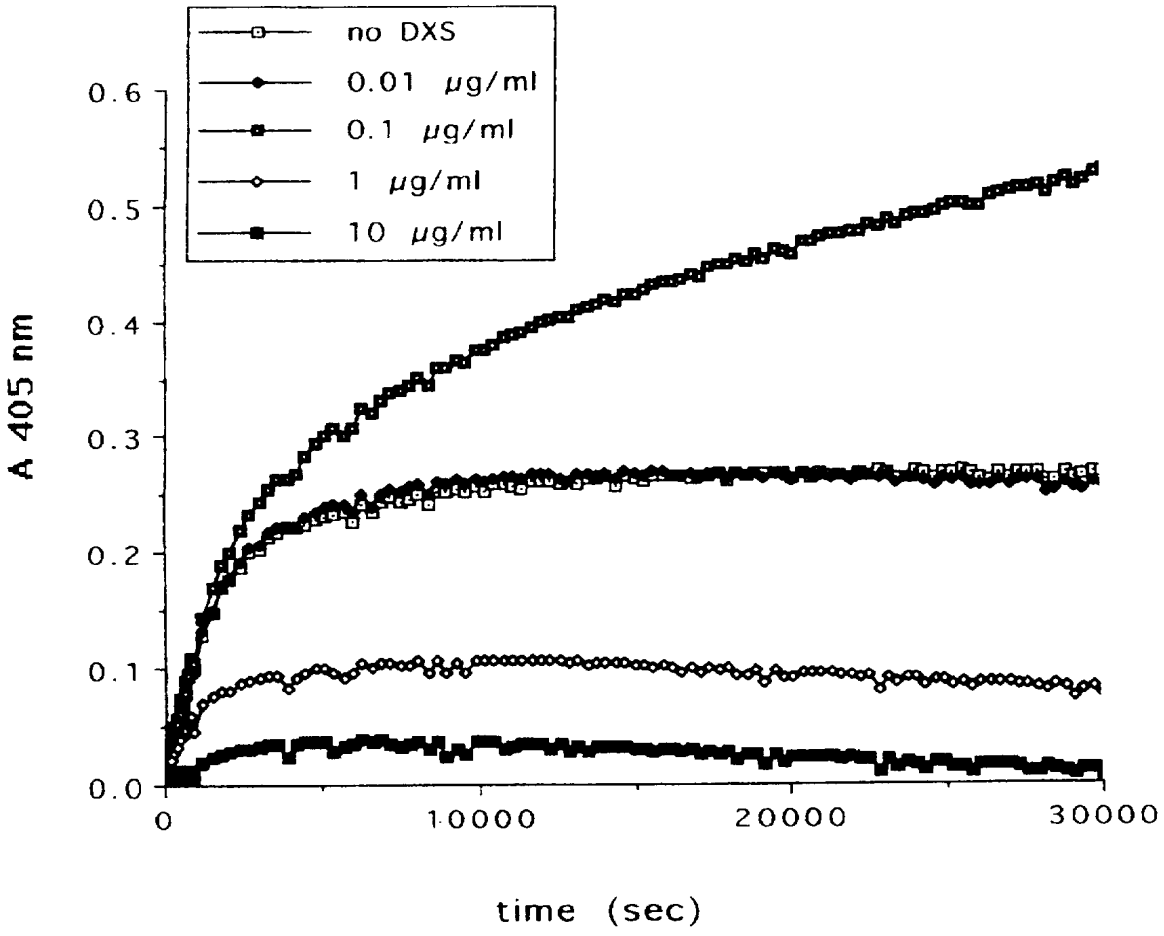
no DXS
0.01 µg/ml
0.1 µg/ml
1 µg/ml
10 µg/ml
A 405 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10000
20000
30000
time (sec)

FIG. 7

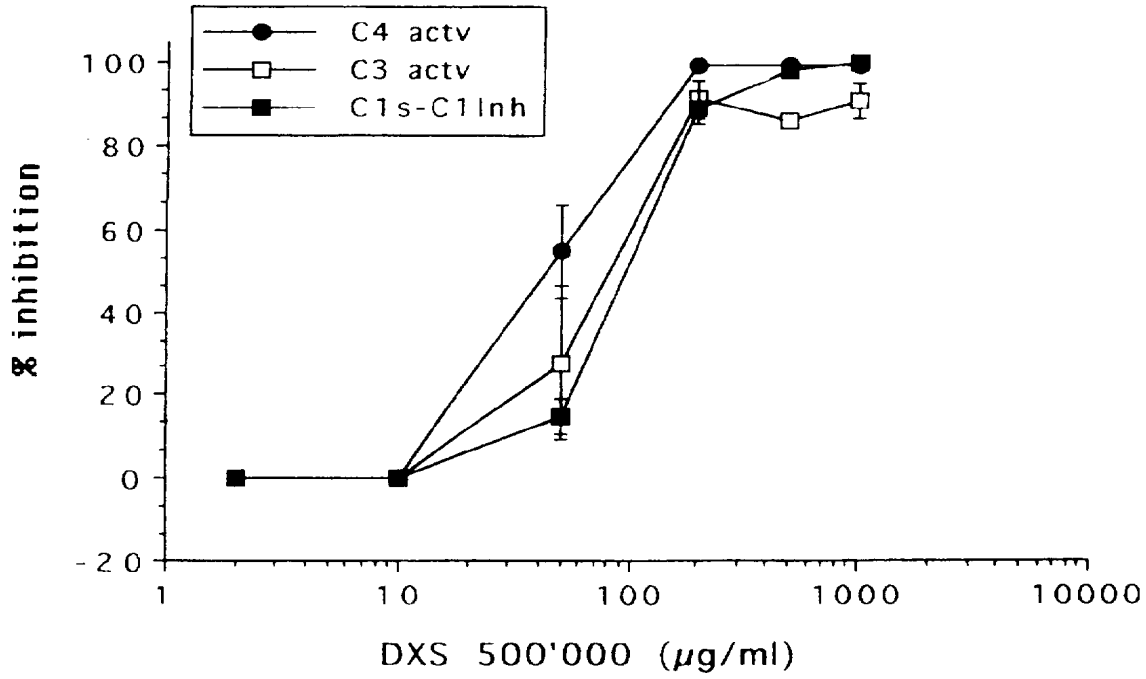
C4 actv
C3 actv
C1s-C1Inh
% inhibition
100
80
60
40
20
0
-20
1
10
100
1000
10000
DXS 500'000 (µg/ml)

FIG. 8

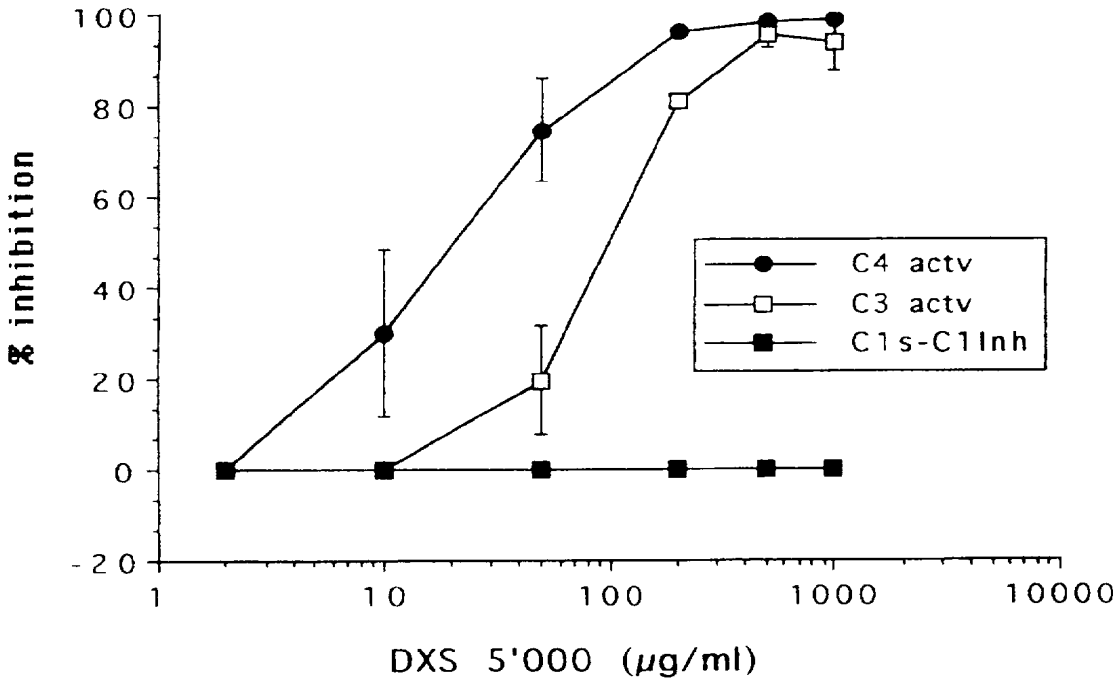
100
80
60
40
20
0
-20
% inhibition
1
10
100
1000
10000
DXS 5'000 (µg/ml)
C4 actv
C3 actv
C1s-C1Inh

FIG. 9

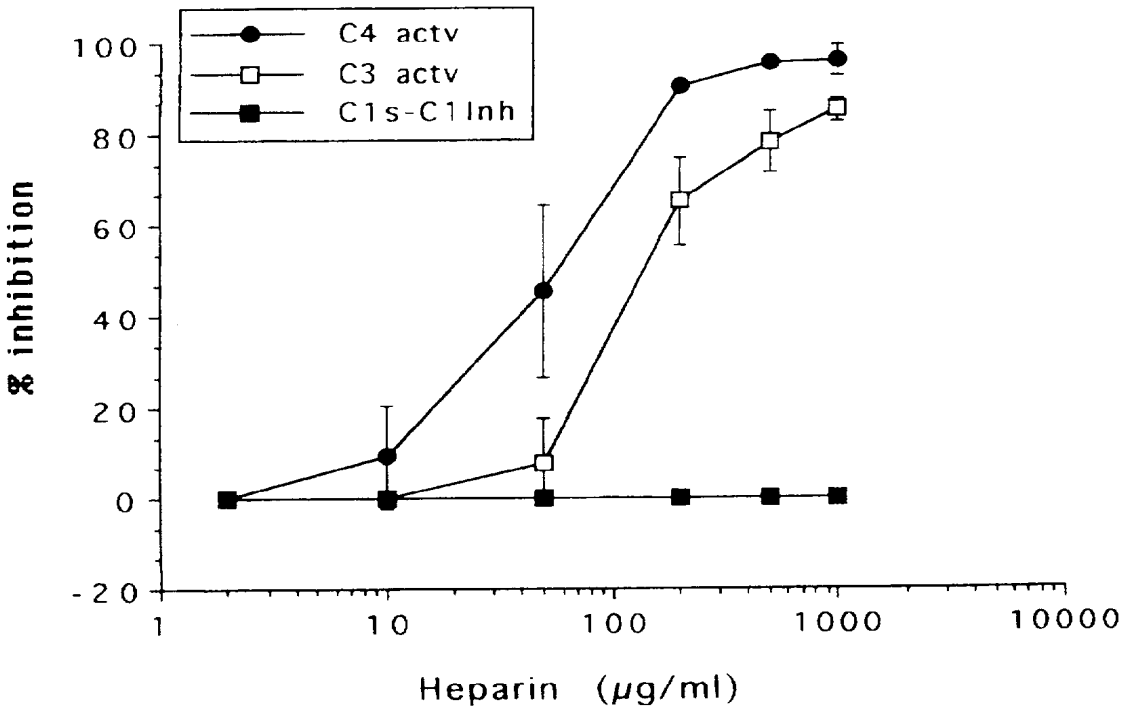
C4 actv
C3 actv
C1s-C1Inh
% inhibition
100
80
60
40
20
0
-20
1
10
100
1000
10000
Heparin (µg/ml)

FIG. 10

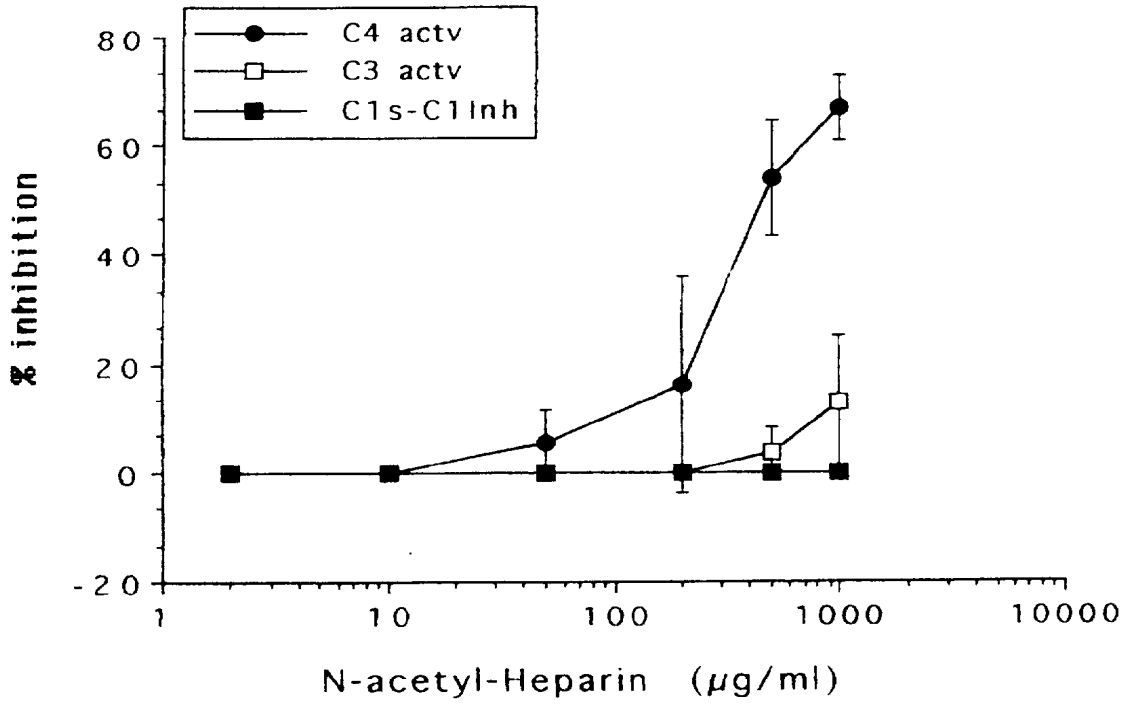
C4 actv
C3 actv
C1s-C1inh
80
60
40
20
0
-20
% inhibition
1
10
100
1000
10000
N-acetyl-Heparin (µg/ml)

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00488

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 88 05301 A (THE AUSTRALIAN NATIONAL UNIVERSITY) 28 July 1988 see claims 1-3,7-9,13,14 see page 5, line 13 - line 14 ---	1,2,4,9, 10,15
X	EP 0 473 564 A (MONSANTO COMPANY) 4 March 1992 cited in the application see claims 1,2,4,5,7 see page 3, line 1 - line 9 ---	1,2,9,15
X	WO 91 05566 A (INVITRON CORPORATION) 2 May 1991 cited in the application	1-11,15
Y	see claims 1-13 --- -/--	16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
12 March 1997	01.04.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Siatou, E

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00488

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 22320 A (GENENTECH INC.) 23 December 1992 cited in the application see page 3, line 34 - page 4, line 7 see claims 1-7	16
X	EP 0 276 370 A (KNOLL AG) 3 August 1988 cited in the application see page 2, line 8 - line 10 see claims 1,2	1-3,9,15
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Y		16
X	DATABASE EMBASE ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL AN: 92306828, M. SAMUEL ET AL: XP002006375 cited in the application & J. Biol. Chem., (1992) 267/27 (19691-19697) see abstract	15
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Information on patent family members

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PCT/NL 96/00488

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